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Cloning, Sequencing, and Expression of the Uroporphyrinogen III Methyltransferase *cobA* Gene of *Propionibacterium freudenreichii* (*shermanii*)

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We cloned, sequenced, and overexpressed *cobA*, the gene encoding uroporphyrinogen III methyltransferase in *Propionibacterium freudenreichii*, and examined the catalytic properties of the enzyme. The methyltransferase is similar in mass (27 kDa) and homologous to the one isolated from *Pseudomonas denitrificans*. In contrast to the much larger isoenzyme encoded by the *cysG* gene of *Escherichia coli* (52 kDa), the *P. freudenreichii* enzyme does not contain the additional 22-kDa peptide moiety at its N-terminal end bearing the oxidase-ferrochelatase activity responsible for the conversion of dihydrosirohydrochlorin (precorrin-2) to siroheme. Since it does not contain this moiety, it is not a likely candidate for synthesis of a cobalt-containing early intermediate that has been proposed for the vitamin B_{12} biosynthetic pathway in *P. freudenreichii*. Uroporphyrinogen III methyltransferase of *P. freudenreichii* not only catalyzes the addition of two methyl groups to uroporphyrinogen III to afford the early vitamin B_{12} intermediate, precorrin-2, but also has an overmethylation property that catalyzes the synthesis of several tri- and tetra-methylated compounds that are not part of the vitamin B_{12} pathway. The enzyme catalyzes the addition of three methyl groups to uroporphyrinogen I to form trimethylpyrrocorphin, the intermediate necessary for biosynthesis of the natural products, factors S1 and S3, previously isolated from this organism. A second gene found upstream from the *cobA* gene encodes a protein homologous to CbiO of *Salmonella typhimurium*, a membrane-bound, ATP-dependent transport protein thought to be part of the cobalt transport system involved in vitamin B_{12} synthesis. These two genes do not appear to constitute part of an extensive cobalamin operon.

Uroporphyrinogen (urogen) III methyltransferase, a key enzyme in the biosynthetic pathways of vitamin B_{12} and siroheme, catalyzes the *S*-adenosyl-L-methionine (SAM)-dependent bismethylation of its substrate, urogen III, resulting in the formation of dihydrosirohydrochlorin (known as precorrin-2 in the vitamin B_{12} pathway). In the biosynthesis of vitamin B_{12} in the anaerobe *Propionibacterium freudenreichii*, labeling experiments have indicated that cobalt is inserted soon after the formation of precorrin-2 (3, 20), and the recent isolation of a cobalt-containing tetramethylated corphinoid, possibly an intermediate, from this organism supports these observations (3). Cobalt insertion in the vitamin B_{12} pathway of the aerobe *Pseudomonas denitrificans*, however, occurs at a much later stage with insertion into hydrogenobyrinic acid diamide (6).

Urogen III methyltransferase has been purified to homogeneity from several different organisms, and the nucleotide sequences of the corresponding genes have been determined, revealing that the enzyme exists in at least two forms. One form, encoded by the *cysG* gene, is required for siroheme and, thus, for cysteine synthesis in *Escherichia coli* (14, 31, 35) and siroheme and vitamin B_{12} synthesis in *Salmonella typhimurium* (8, 10). The *S. typhimurium* enzyme is closely related to the *E. coli* enzyme (90% identity and 95% similarity). The second form, encoded by the *cobA* gene, is required for vitamin B_{12} synthesis in *P. denitrificans* (1, 5, 7) and has also been isolated from *Bacillus megaterium* (25) and *Methanobacterium ivanovii* (2). Both forms of the enzyme perform the in vivo synthesis of precorrin-2, but in addition to its methylase activity, CysG (but not CobA) has NAD^+-dependent precorrin-2 oxidase and ferrochelatase activities (31). Thus, CysG is believed to be a multifunctional enzyme solely responsible for the synthesis of siroheme (31, 33) in *E. coli*.

CysG and CobA can be differentiated by their physical and enzymatic characteristics. The CysG enzyme is composed of 458 amino acids with a mass of ~52 kDa, whereas the smaller CobA protein is composed of 280 amino acids with a mass of ~30 kDa and is homologous only to the C-terminal region of CysG (amino acids 210 to 458). Thus, the N-terminal portion of CysG is believed to contain the NAD^+-dependent precorrin-2 oxidase and ferrochelatase activities (31). CobA performs only methylation of C-2 and C-7 of urogen III or its isomer, urogen I (1), whereas CysG performs these methylations and also has an overmethylation property. Previous studies with CysG have resulted in the in vitro synthesis of several tri- and tetra-methylated compounds such as trimethylpyrrocorphins derived from urogen I and urogen III (30, 36), a tetramethylpyrrocorphin derived from precorrin-3 (27), and factor S3 (22), a natural product previously isolated from *P. freudenreichii*.

Experiments with partially purified extracts from *P. freudenreichii* provided the earliest evidence that urogen III methyl-

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transferase catalyzes both methylation steps to form precorrin-2 from urogen III (17). Since CysG has been shown to insert not only iron but also cobalt into factor II, it has been suggested (31) that urogen III methyltransferase in *P. freudenreichii* may also be a CysG-like enzyme that could synthesize a cobalt-containing analog of siroheme as an early intermediate. To determine the form of urogen III methyltransferase in *P. freudenreichii*, we cloned, sequenced, and expressed its gene and examined the catalytic properties of the enzyme.

MATERIALS AND METHODS

Chemicals and enzymes. [^{13}C]aminolevulinic acid ([^{13}C]ALA) isotopomers (12, 24) and *S*-adenosyl-[$^{13}CH_3$]-L-methionine (9) were prepared as previously described. All other chemicals were purchased from Sigma Chemical Co. and were of the highest grade obtainable. ALA dehydratase, porphobilinogen deaminase, urogen III synthase (cosynthetase), CysG, and precorrin-2 methyltransferase were isolated from recombinant strains of *E. coli* as previously described (22, 26, 27, 34). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs or Boehringer Mannheim and used as directed by the supplier. Other molecular biology techniques were performed according to standard procedures (15).

Isolation of the *P. freudenreichii* gene encoding urogen III methyltransferase. The *P. freudenreichii* gene for urogen III methyltransferase was isolated by transformation of an *E. coli cysG* mutant (*E. coli* CBK103(F$^-$) *cysG*::Tn*5* *thy*, provided by M. G. Marinus, University of Massachusetts Medical School) with a library that had been constructed by insertion of a partial *Sau*3AI digest of *P. freudenreichii* genomic DNA into the *Bam*HI site of plasmid pUC18 (21). The transformed cells were plated on M9 minimal agar plates containing thymine (4 μg/ml), ampicillin (50 μg/ml), and kanamycin (50 μg/ml), and incubated at 37°C, and the resulting colonies were restreaked on selective medium. Plasmids isolated from strains that survived the second round of selection were characterized by restriction analysis and transformed into *E. coli* TB1 for routine maintenance and storage as frozen permanent stocks.

DNA sequence and protein analysis. Plasmid DNA was sequenced in both directions by the dideoxy chain termination method (29). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (13). Whole-cell samples for SDS-PAGE analysis were prepared by centrifuging 1 ml of cell culture in a microcentrifuge, resuspending the cells in 200 μl of sample buffer, and heating in a boiling water bath for 5 min. Protein concentrations were determined by the dye-binding assay of Bradford (4).

Growth of cells and preparation of cell lysates. A single colony of the appropriate strain was inoculated into 50 ml of Luria-Bertani (LB) medium containing 50 μg of ampicillin per ml. When this culture had attained mid-log growth, it was used to inoculate (10 ml/liter) one liter of LB-ampicillin in 2.8-liter Fernbach flasks which were incubated overnight (16 to 18 h) in a New Brunswick model G25 environmental shaker set at 37°C and 250 rpm. The cells were collected by centrifugation, resuspended in 20 ml of buffer (100 mM Tris-HCl, pH 8.0) per liter of culture, and incubated for 30 min at room temperature with 50 μg of lysozyme per ml. The cells were lysed by sonication (3 times for 45 s) on ice, maintaining the temperature below 20°C. Unlysed cells and cell debris were removed by centrifugation at 12,000 × *g* for 10 min, and the supernatant, hereafter called the cell lysate, was tested for methyltransferase activity.

Synthesis of urogen I and urogen III substrates. Urogen I and urogen III were synthesized from ALA in a multienzyme reaction with enzymes isolated from recombinant strains of *E. coli* as previously described (16, 26). Briefly, ALA (3 to 5 mg) was first converted to porphobilinogen in a reaction mixture containing 100 ml of 0.1 M Tris-HCl, pH 8.0, and 15 mg of ALA dehydratase. After incubation at 30°C for 1 h, the solution was degassed (three cycles of freeze-thaw under vacuum) and transferred to an argon-purged glove box (<5 ppm of O_2), where it was supplemented with 2 mg of lyophilized porphobilinogen deaminase for the synthesis of urogen I or with 2 mg of porphobilinogen deaminase plus 2 mg of lyophilized urogen III synthase for the synthesis of urogen III. Since the urogens, precorrins, and pyrrocorphins are all oxygen sensitive and can be handled only under anoxic conditions, all solutions were degassed and the subsequent incubations and product isolation were performed in the glove box.

Assay of methyltransferase activity. Methyltransferase activity was assayed by ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectroscopic analysis of the products from a reaction mixture containing cell lysate, SAM, and ^{13}C-labeled substrate (urogen I or urogen III, derived from ^{13}C-labeled ALA). The urogen-generating system described above was supplemented with 20 mg of SAM and 40 ml of cell lysate (5 mg of total protein per ml) prior to degassing and addition of the other enzyme(s). The solution was degassed as above and transferred to an argon-purged glove box. The other enzyme(s) was added, and the solution was incubated at ambient temperature. The tetrapyrrolic products were adsorbed onto a small DEAE-Sephadex column, washed with 0.3 M KCl, and eluted from the column with 20% D_2O in 2.0 M KCl. The structures of the compounds were determined by comparison of their ^{13}C-NMR spectra to those of previously isolated tetrapyrroles (22, 27, 30, 34, 36).

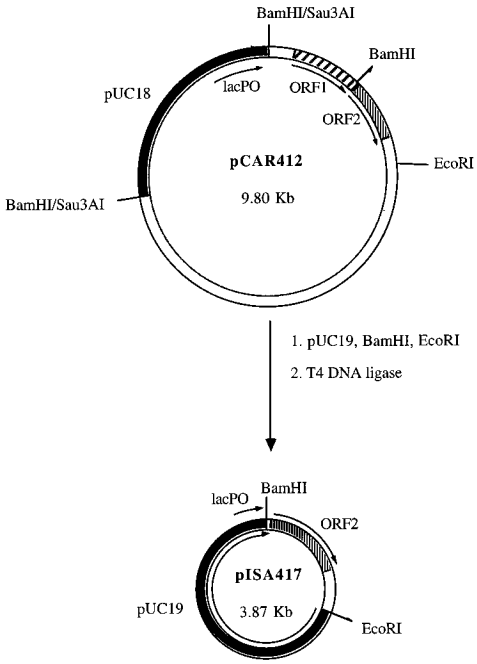

FIG. 1. Structure of plasmid pCAR412, containing 7.1 kb of *P. freudenreichii* genomic DNA in pUC18, and construction of pISA417, containing only the *cobA* gene for expression of *P. freudenreichii* urogen III methyltransferase. lacPO, *lac* promoter-operator region.

RESULTS

Isolation of the *P. freudenreichii* gene encoding urogen III methyltransferase. Transformation of an *E. coli cysG* mutant with a *P. freudenreichii* genomic library yielded several colonies that grew on minimal medium. Restriction analysis of plasmids isolated from these colonies revealed that they contained a 9.8-kb plasmid (pCAR412) consisting of pUC18 with a 7.1-kb *P. freudenreichii* genomic DNA insert (Fig. 1). *E. coli* strains bearing this plasmid, when grown on enriched medium (LB-ampicillin), displayed a bright red fluorescence under UV light (302 nm) that was not observed when these strains contained pUC18 alone. Similar fluorescence was observed when cells bearing plasmids that overexpress CobA or CysG were grown on LB-ampicillin. The fluorescence has been shown (unpublished results) to be due to the cytoplasmic accumulation of trimethylpyrrocorphin and oxidized precorrin-2 (factor II) in these strains.

Nucleotide sequence of the *P. freudenreichii cbiO* and *cobA* genes. The nucleotide sequence of 2.5 kb of pCAR412 distal to the *lac* promoter-operator region of pUC18 was determined (Fig. 2), revealing two open reading frames (ORFs). The first ORF (bases 329 to 1222) encoded a protein of 297 amino acids with a predicted mass of 31,244 Da. A search with the BLAST program revealed homology (37% identity, 61% similarity) between this protein and the *cbiO* gene product from *S. typhimurium*, a membrane-associated, ATP-dependent transport protein thought to be involved in cobalt import for use in cobalamin biosynthesis (28). The second ORF (bases 1219 to 1992) encoded a protein of 257 amino acids with a predicted mass of 27,080 Da that displayed homology to CorA, CobA, and several other known or predicted urogen III methyltransferases, including the carboxyl-terminal region of CysG.

Overexpression of the *cobA* gene product. TB1 cells bearing pCAR412 overexpressed a protein with an M_r of 27,000, and lysates of these cells demonstrated urogen III methyltrans-

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   1  GGATCGCCTCGCTCACCTACCGGCTGCTGTTCGTGCTGGCCGAGACCACCGCCAATGTGCTGGCCGCGCGCAACGCTGCCGGCTCGGGGACAACCCGGTGGGCCGGTGGAACGGCCTGTCGC
 121  GGCGCTGGCACAACACGGCCGCTGCCGTGGGGGCCATCGGCCTGCGCGCCTGGGACCGCTCGTCGCGGCTCACCGAGGGGCTGGCCCATCGTGGCTTCGAGACCTCCTTGGTGACGCTGC
                                                                                                  M  S  A  L  L  A  A  H  D  L  V
 241  CCGTGCCCCGGGTCGCCTCGCCGCGCCTGATCACCGCCACCGTCGTGGTGCTGGCCGCAATCTGGTCGATCAGCCTGGTGGTGGCCCGATGAGCGCCCTGCTGGCCGCCCACGACCTCGT
       A  G  F  P  D  R  P  R  V  L  D  T  V  N  L  A  I  G  R  G  T  R  L  A  L  L  G  A  N  G  S  G  K  T  T  L  L  S  C  L
 361  GGCCGGCTTCCCCGATCGTCCCCGCGTGCTCGACACGGTGAACCTCGCCATCGGCCGCGGCACCCGGCTCGCGCTGCTCGGCGCCAACGGCTCGGGCAAGACCACCCTGCTCAGCTGCCT
       A  G  S  L  K  P  T  G  G  A  V  E  R  D  G  A  P  I  D  W  G  R  K  G  L  R  E  H  R  R  H  V  Q  M  V  L  Q  D  P  D
 481  GGCGGGCTCGCTGAAGCCCACCGGCGGTGCCGTCGAACGCGATGGCGCCCCCATCGACTGGGGACGCAAGGGACTGCGCGAGCACCGCCGCCATGTGCAGATGGTGCTGCAGGACCCCGA
       D  Q  L  F  S  A  D  V  S  Q  D  V  S  F  G  P  M  N  L  G  L  E  P  D  E  V  H  A  R  V  R  E  A  L  T  L  L  G  A  D
 601  TGACCAGCTGTTCAGCGCCGACGTCTCACAGGACGTGTCGTTCGGTCCGATGAACCTGGGGTTGGAGCCCGACGAGGTGCATGCCCGGGTGCGTGAGGCCCTCACGCTGCTGGGCGCCGA
       H  L  A  E  R  A  T  H  Q  L  S  Y  G  E  R  K  R  V  A  T  A  G  A  V  A  M  R  P  D  V  L  M  L  D  E  P  T  A  G  L
 721  CCACCTGGCCGAACGCGCCACCCACCAGCTGTCCTACGGCGAACGCAAGCGCGTCGCCACCGCCGGCGCCGTGGCGATGCGTCCCGATGTGCTCATGCTCGATGAGCCGACCGCCGGACT
       D  P  L  G  V  H  Q  M  R  D  A  L  D  R  L  T  D  A  G  T  T  I  V  M  A  T  H  D  V  A  L  A  L  D  W  A  D  E  V  A
 841  CGATCCGCTGGGCGTGCACCAGATGCGTGACGCGCTCGACCGGCTCACCGACGCCGGCACGACCATCGTCATGGCCACCCACGATGTGGCGCTGGCCCTCGACTGGGCCGACGAGGTCGC
       I  V  C  D  A  T  V  H  Q  G  A  P  E  A  L  L  S  D  A  D  L  V  A  R  S  H  L  D  T  P  W  P  L  A  L  A  A  A  L  G
 961  TATCGTCTGTGACGCGACGGTGCACCAGGGAGCTCCCGAGGCCCTGCTCTCCGACGCCGACCTGGTGGCGCGCAGCCATCTGGACACGCCCTGGCCGTTGGCACTTGCCGCCGCCCTGGG
       L  S  A  R  P  R  N  L  D  E  A  V  A  A  L  A  P  A  L  G  E  F  A  A  T  H  D  A  A  A  S  V  P  G  S  D  D  T  T  N
1081  ACTGTCCGCCCGTCCACGCAACCTCGACGAGGCGGTGGCCGCACTGGCTCCCGCGCTCGGCGAGTTCGCCGCCACGCACGACGCCGCCGCATCCGTGCCCGGATCCGACGACACCACCAA
       T  D  E  E  T  R  *
                        M  T  T  T  L  L  P  G  T  V  T  L  V  G  A  G  P  G  D  P  E  L  V  T  V  A  G  L  R  A  V  Q  Q  A
1201  CACCGATGAGGAAACCCGATGACCACCACACTGTTGCCCGGCACTGTCACCCTCGTCGGCGCCGGGCCCGGCGACCCTGAACTCGTCACCGTGGCCGGCCTGCGGGCCGTGCAGCAGGCC
       E  V  I  L  Y  D  R  L  A  P  Q  D  L  L  S  E  A  S  D  D  A  E  L  V  P  V  G  K  I  P  R  G  H  Y  V  P  Q  E  E  I
1321  GAGGTGATCCTCTACGACCGGCTCGCCCCGCAGGACCTGCTGTCGGAGGCGTCCGACGACGCCGAACTCGTGCCGGTCGGCAAGATCCCGCGCGGCCACTATGTGCCCCAGGAGGAGATC
       N  Q  L  L  V  A  H  A  R  E  G  R  K  V  V  R  L  K  G  G  D  S  F  V  F  G  R  G  G  E  E  W  Q  A  C  A  E  A  G  I
1441  AACCAACTGCTCGTCGCGCACGCCCGCGAGGGCCGCAAGGTGGTGCGCCTCAAGGGTGGCGACTCGTTCGTCTTCGGGCGTGGCGGCGAGGAATGGCAGGCCTGCGCCGAGGCCGGCATC
       P  V  R  V  I  P  G  V  S  S  A  T  A  G  P  A  L  A  G  I  P  L  T  H  R  H  L  V  Q  G  F  T  V  V  S  G  H  V  S  P
1561  CCGGTGCGCGTGATCCCGGGAGTCTCCTCGGCCACCGCGGGCCCGGCGCTGGCCGGCATCCCGCTGACCCATCGCCACCTGGTGCAGGGGTTCACCGTCGTGTCGGGGCATGTATCGCCC
       S  D  E  R  S  E  V  P  W  R  Q  L  A  K  D  R  L  T  L  V  I  L  M  G  V  A  H  M  R  D  I  A  P  E  L  M  A  G  G  L
1681  AGCGACGAGCGCTCCGAGGTGCCATGGCGCCAACTCGCCAAGGACCGGCTCACGCTGGTGATCCTGATGGGCGTGGCCCATATGCGCGACATCGCGCCGGAATTGATGGCCGGCGGGCTG
       P  A  D  T  P  V  R  V  V  S  N  A  S  L  A  S  Q  E  S  W  R  T  T  L  G  D  A  V  A  D  M  D  A  H  H  V  R  P  P  A
1801  CCTGCCGACACCCCCGTGCGCGTGGTGAGCAATGCGAGCCTGGCCAGCCAGGAATCGTGGCGCACCACGCTGGGCGATGCCGTGGCCGACATGGACGCGCACCACGTGCGTCCGCCCGCG
       L  V  V  V  G  T  L  A  G  V  D  L  S  H  P  D  H  R  A  P  S  D  H  *
1921  CTGGTGGTGGTGGGTACCCTGGCCGGCGTCGACCTGTCGCATCCCGACCATCGCGCGCCCAGCGACCACTGAGTCCCGGCCGGCCGCCGGGCACCTGCTGATCGATCGGCGTGGCCTGCG
2041  GCCGTGAGACACTGCCGAACGTGTTTGACCTCGATCGGATAGCCGCCGGGCTGCCGGTTGGTGGGCGCCTGGACGAGCTCGCCGCGGCCCTCACGGCGCCGGATGCGCGCCTGGTGGTGC
2161  AGGCCCCTCCGGGCTCGGGCAAGACCACCGTGATGCCGCCCCTGGCCGCGCATCACCATGCCGGGCGCGTCGTGGTCACCCAACCCCGGCGCATCGCCGCCCGCGCGGCGGCCCACCGAC
2281  TCGCCGAACTGTCCGGCACCGCCCTGGGCCGCGAGGTCGGCTTCACGGTGCGCGGGGAGTCACGCCGATCGGATGCCACGCGCGTCGAATTCGTGACCACCGGCGTGCTGGTCAACCGCG
2401  TATTGCGTGACCCCGAGCTCGCCGGGGTGGGCACGGTGATCCTCGACGAGGTGCACGAGCGTCGCCTGGACACCGACCTGGCGTTCGCGATGGTGCATGA
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FIG. 2. Nucleotide sequence of 2.5 kb of pCAR412, starting with the promoter-proximal *Bam*HI/*Sau*3AI junction. The amino acid sequences shown are derived from the translations of ORF-1 (bases 329 to 1222; *cbiO*) and ORF-2 (bases 1219 to 1992; *cobA*). The *Bam*HI (position 1181) and *Eco*RI (position 2367) cleavage sites used for subcloning ORF-2 into pUC19 are underlined.

ferase activity. However, for further enzymatic studies, it was desirable to use a plasmid in which urogen III methyltransferase was the only possible expressed protein. Therefore, a plasmid containing ORF-2 as the only complete gene (pISA417) was constructed by subcloning the 1.2-kb *Bam*HI-*Eco*RI fragment from pCAR412 into pUC19 (Fig. 1). TB1 cells bearing this plasmid were fluorescent when grown on LB-ampicillin, and SDS-PAGE analysis of whole cells of TB1 (pISA417) revealed the overexpression of a protein with an M_r of 27,000 (Fig. 3). After centrifugation at 100,000 $\times$ *g* for 15 min, the overexpressed protein was found in the cell lysate (not shown), indicating that the protein was soluble and not contained in inclusion bodies.

Methyltransferase activity of the *cobA* gene product. Cell lysates of TB1(pISA417) containing the overexpressed protein were assayed for methyltransferase activity under various conditions with either ^{13}C-labeled urogen I or ^{13}C-labeled urogen III as substrate. NMR analysis of the isolated products revealed that *P. freudenreichii* urogen III methlytransferase has the same methylation activities (Fig. 4) as those previously described for the *E. coli* CysG enzyme, including the overmethylation activity that is not observed with the *P. denitrificans* CobA enzyme. Control lysates of TB1(pUC19), which presumably have a low level of endogenous CysG expressed from the chromosomal gene, had no urogen III methyltransferase activity detectable by the NMR assay even after a 15-h incubation. The best substrates for analysis of urogen III methyltransferase activity are urogens derived from [3-^{13}C]ALA in which the carbons that become methylated are ^{13}C labeled (Fig. 4) and appear in a ^{13}C-NMR spectrum in the sp^2 region as a single peak at around 120 ppm. The addition of a methyl group to one of these carbons converts it to an sp^3 center with a concomitant upfield shift to 40 to 60 ppm. Thus, after a 4-h incubation of a TB1(pISA417) lysate with unlabeled SAM and urogen III derived from [3-^{13}C]ALA, ^{13}C-NMR spectroscopy of the product revealed the presence of two new sp^3 carbons (Fig. 4A), which is consistent with the addition of two methyl groups to urogen III. Complete NMR data of the product derived from [4-^{13}C]ALA and [5-^{13}C]ALA were identical to those previously reported for precorrin-2 (32), thus proving that the isolated gene does, indeed, encode urogen III methyltransferase necessary for the synthesis of precorrin-2. Incu-

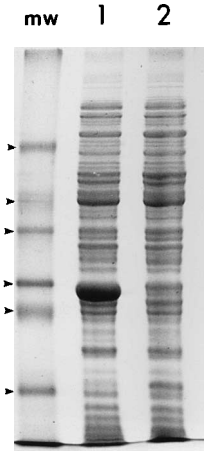

FIG. 3. SDS-PAGE analysis of whole-cell lysates of *E. coli* TB1 bearing pISA417 (lane 1) and, as a control, TB1 bearing pUC19 (lane 2). The molecular weight markers (arrows) are, from the top, bovine serum albumin (66,000), egg albumin (45,000), glyceraldehyde-3-*P*-dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), and trypsin inhibitor (20,000).

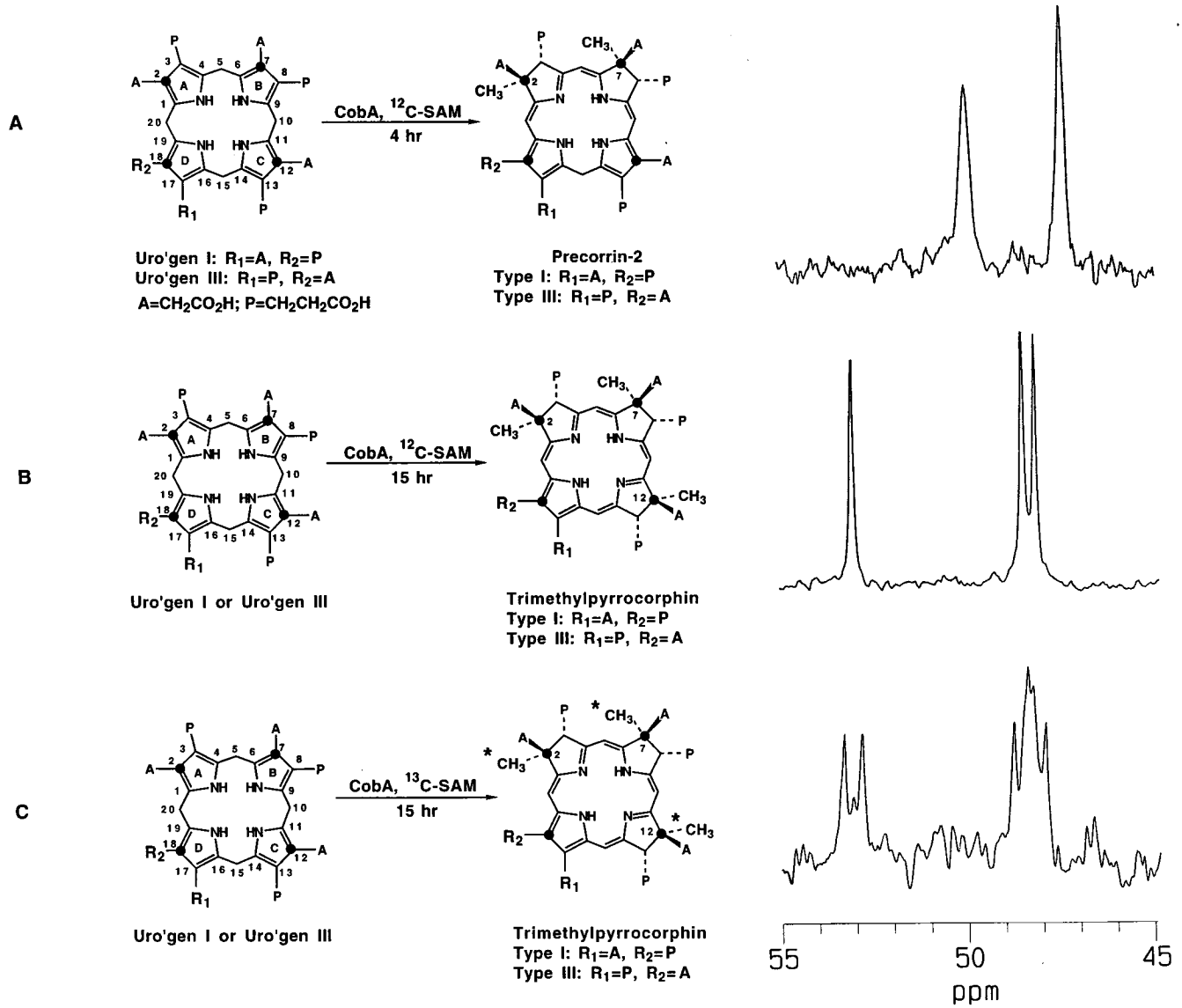

FIG. 4. Selected regions of ^{13}C-NMR spectra showing the methylase activity of urogen III methyltransferase (CobA) from *P. freudenreichii*. Shown are the spectra of the products isolated after urogen III derived from [3-^{13}C]ALA was incubated with CobA and [^{12}C]SAM for 4 h (A), with CobA and [^{12}C]SAM for 15 h (B), and with CobA and [^{13}C]SAM for 15 h (C). The products were isolated and analyzed by ^{13}C-NMR spectroscopy as described in Materials and Methods. Note that in urogen III the D pyrrole ring is inverted with respect to the D ring of urogen I, resulting in reversal of the acetate and propionate side chains of this ring.

bation of labeled urogen III and unlabeled SAM with *P. freudenreichii* CobA for a longer time (15 h) afforded three sp^3 carbon centers (Fig. 4B), consistent with the addition of three methyl groups. Confirming evidence for the addition of three methyl groups was provided by substituting ^{13}C-methyl-labeled SAM for unlabeled SAM in this reaction, which resulted in a spectrum containing three doublets (Fig. 4C), corresponding to $^{13}CH_3$-^{13}C coupling between the newly added methyl groups and the quaternary carbons at the base of the acetyl groups. Complete NMR analysis of this product showed it to be identical to the trimethylpyrrocorphin previously synthesized with CysG (36). Urogen I also served as a substrate for *P. freudenreichii* CobA, with the formation of type I precorrin-2 after 4 h and type I trimethylpyrrocorphin after a prolonged incubation. The NMR spectra of these type I compounds are identical to those derived from urogen III. We previously reported the formation of a tetramethylated compound when CysG was incubated with precorrin-3 (27). This compound is not an intermediate in the vitamin B_{12} pathway and has not yet been fully characterized, but it was of interest to test if *P. freudenreichii* CobA as well as CysG could use precorrin-3 as a substrate. Thus, precorrin-2 methyltransferase (CobI, which methylates precorrin-2 at C-20 to form precorrin-3) (32) was added to an incubation mixture containing urogen III derived from [3-^{13}C]ALA, SAM, and the TB1(pISA417) lysate. The product isolated from this reaction mixture had an NMR spectrum identical to that of the tetramethylated compound formed by CysG under similar conditions. Thus, it is apparent that *P. freudenreichii* CobA performs all of the methylations with all of the substrates previously reported for CysG.

Nucleotide sequence accession number. The nucleotide sequences of the *P. freudenreichii cbiO* and *cobA* genes have

been submitted to GenBank and assigned accession number U13043.

DISCUSSION

We have reported the cloning, sequencing, and overexpression of the gene encoding urogen III methyltransferase in *P. freudenreichii (shermanii*). The *P. freudenreichii* methyltransferase has high similarity to *P. denitrificans* CobA and to the carboxyl-terminal portion of *E. coli* CycG. Homology with several other confirmed or proposed methyltransferases was found, none of which contain the additional oxidase-ferrochelatase region of CysG. Hence, the gene associated with the *P. freudenreichii* methyltransferase has been called *cobA* rather than *cysG*. Among all 10 organisms from which urogen III methyltransferase has been described at either the gene level or the protein level (references 2, 5, 11, 25, and 34 and for unpublished sequences, GenBank accession numbers D28503, U05002, U08566, Z28294), the combination of urogen III methyltransferase with precorrin-2 oxidase and ferrochelatase into a single enzyme is unique to *E. coli* and its close relative, *S. typhimurium*. Lack of the oxidase-chelatase portion implies that urogen III methyltransferase is not a likely candidate for cobalt insertion for the synthesis of an early vitamin B_{12} intermediate in *P. freudenreichii*. One possibility is that cobalt insertion occurs spontaneously, since porphyrinoids are known metal chelators. Indeed, we have recently shown that factor S3 can be formed by nonenzymatic complexation of zinc into its metal-free tetramethylated precursor (22). We cannot rule out, however, the existence of a separate gene encoding an enzyme with properties similar to those of the N-terminal portion of CysG.

Surprisingly, the methylation properties of urogen III methyltransferase of *P. freudenreichii*, unlike CobA from *P. denitrificans*, cover the complete repertoire of those previously reported for CysG. Not only does urogen III methyltransferase synthesize precorrin-2 found in the siroheme and vitamin B_{12} pathways, it also catalyzes the addition of three methyl groups to urogen I to form the trimethylpyrrocorphin intermediate necessary for the biosynthesis of the zinc-containing natural products, factors S1 and S3, previously isolated from *P. freudenreichii* (18, 19).

Analysis of possible peptides encoded by the DNA upstream from the *cbiO* gene and downstream from the *cobA* gene did not reveal any protein homologous to the NH_2-terminal portion of CysG or any other siroheme, vitamin B_{12}, or nitrite reductase biosynthetic enzyme. Thus, the inclusion of *cobA* in a larger operon involved in the biosynthesis of one of these metabolites, as is observed in other organisms (5, 11, 23), is not likely in *P. freudenreichii*. With the genomic library of *P. freudenreichii* now available (21), the search for the genes encoding the remaining vitamin B_{12} and siroheme enzymes is in progress.

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